

Maintenance of Episomal SV40 Genomes in GM637 Human Fibroblasts

Kuan-Chun Huang,*† Edith F. Yamasaki,*† and Robert M. Snapka*††¹

*Department of Radiology and †Department of Molecular Virology, Immunology, and Medical Genetics and ‡The Ohio State University Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210

Received June 3, 1999; returned to author for revision July 13, 1999; accepted August 3, 1999

Episomal SV40 (SV40: simian virus 40, *Polyomavirus maccacae*) has been reported in SV40-transformed human fibroblast cell lines the integrated SV40 sequences of which are unlikely to give rise to episomal copies by recombinational mechanisms. The levels of episomal viral DNA in these lines are high, being easily visualized by ethidium staining of agarose gels after electrophoresis. We find that the episomal mutant gmSV40 in GM637 cells represents a persistent lytic infection that can be cured by treatment with neutralizing antibody, leaving only the chromosomally integrated viral genomes. The finding that maintenance of the gmSV40 in GM637 cells is due to persistent infection raises a note of caution for SV40-transformed lines with episomal SV40 genomes because these lines often are used in studies of DNA replication and repair. An infective center assay that does not depend on plaque formation shows that gmSV40 is a host range mutant, with poor infectivity for CV-1 monkey kidney cells and greatly increased infectivity for human cells. Passage of gmSV40 through monkey kidney cells selects for variants with greatly increased infectivity for monkey cells and, independently, for cytopathic variants that produce plaques. Thus plaque assays can give very unreliable infective center values in studies of host range mutants. © 1999 Academic Press

INTRODUCTION

Simian virus 40 (SV40) is a small DNA tumor virus that was discovered in Rhesus monkey kidney cells (Sweet and Hilleman, 1960). Although SV40 did not cause detectable cytopathic effects in infected Rhesus monkey cells, a pronounced cytopathic effect including vacuolation and cell death was observed in SV40-infected African green monkey kidney cells (Sweet and Hilleman, 1960). In human cells, the ability to support efficient lytic infection of SV40 is varied. Inefficient SV40 infection has been reported in human fibroblast and human embryonic kidney cells (Zouzas *et al.*, 1980; Ozer *et al.*, 1981; Tooze, 1981; Neufeld *et al.*, 1987; O'Neill *et al.*, 1998), while efficient lytic growth of SV40 has been reported in human spongiblasts, human foetal neural cells and some human tumor cell lines (O'Neill and Carroll, 1981; O'Neill *et al.*, 1998). It also has been shown that SV40 can persist for ≤ 58 days in human kidney cells without detectable cytopathic effect (Hsiung and Gaylord, 1961). There is evidence that early steps in infection, such as attachment, internalization, or uncoating are limiting in SV40 infections of some human cells with the levels of viral DNA replication being high in the fraction of cells successfully infected (Ozer *et al.*, 1981).

SV40-transformed human fibroblast lines such as

GM637 and XP12ROSV (a xeroderma pigmentosum line) often have been used in studies of DNA replication and repair, the transformed phenotype, and cell-cycle control (Simon *et al.*, 1981; Schwartz *et al.*, 1982; Domoradzki *et al.*, 1984; Dusinska and Slamenova, 1992; Fritz and Kaina, 1992; Denissenko *et al.*, 1994; Mitchell *et al.*, 1994; Wright *et al.*, 1998). Both lines have been reported to maintain high levels of episomal SV40 genomes that replicate as stable plasmids that are carried through the cell cycle (Maulbecker *et al.*, 1992). The episomal SV40 genomes in GM637 cells (gmSV40) and in XP12ROSV cells (xpSV40) differ from wild-type SV40 genomes in having an identical deletion of 323 bp (4643–4966) that spans the splice donor site of the first large T antigen intron. This prevents the production of small t antigen and results in a large T antigen that is smaller by 14 amino acids (Maulbecker *et al.*, 1992). GmSV40 and xpSV40 also share an identical point mutation which changes a C to an A at nucleotide 4642 (in the A gene encoding large T antigen) and similar but not identical changes in the enhancer region. The A gene deletion has been reported to give gmSV40 chromosomes a replication advantage in human cells (Maulbecker *et al.*, 1992). Density-labeling studies have suggested that replication of the episomal SV40 in GM637 cells is not of the lytic or random choice type but also not completely under cellular replication control (Maulbecker, 1989). The replicating SV40 genomes in GM637 cells also have been reported to be very homogeneous in size and free of “defective” or “variant” viral genomes (Maulbecker, 1989); this might be expected to accumu-

¹ To whom reprint requests should be addressed. Fax: (614) 292-7237. E-mail: snapka.1@osu.edu.

late due to occasional replication errors in either a persistent lytic infection or in continual high level episomal replication.

Tandemly integrated, intact copies of SV40 are thought to give rise to episomal virus in SV40-transformed human cell lines by rare recombination events (Tooze, 1981). The episomal SV40 genomes in GM637 and XP12ROSV do not seem to be produced by excision of the integrated copies because excision is thought to require tandemly repeated intact copies of the viral genome. The integrated SV40 copies in GM637 cells are located on chromosome 8, and evidence from Southern blotting experiments suggest that this site contains two copies of SV40 separated by host DNA with at least one of the copies containing a large deletion (Hwang, 1980). Likewise there is evidence that the proviral sequences in XP12ROSV are replication incompetent and thus cannot generate episomal copies (Royer-Pokora *et al.*, 1984).

Our laboratory uses SV40 as a model mammalian replicon in studies of aberrant DNA replication caused by drugs that target enzymes of DNA replication (Snapka and Permana, 1993; Snapka *et al.*, 1996; Woo *et al.*, 1999). The extension of the SV40 model system to human cells was hampered by the limited infectivity of wild-type SV40 for many human cell lines. Thus we turned to GM637 cells because of the reported high levels of episomal SV40 DNA replication in this cell line.

We initiated these studies by subcloning the GM637 cell line to ensure homogeneity. The finding, reported here, that most subclones were free of episomal virus suggested the possibility of a persistent but inefficient infection. We show here that maintenance of the episomal SV40 genomes in GM637 cells requires continual reinfection. The GM637 cells can be "cured" of episomal SV40 by treatment with neutralizing antibody or by subcloning. We also show that the large T antigen deletion present in the episomal virus is not present in the integrated proviral copies, proving that episomal virus does not arise by excision of these copies. The "episomal virus" phenotype can be reestablished in episome-free cells by infection with gmSV40 or wild-type SV40 viral stocks. Because gmSV40 does not plaque efficiently on monkey cells and because our human cell lines did not produce visible plaques with either wild-type SV40 or gmSV40, we developed an infective center assay that does not depend on cytopathic effect. Using this infective center assay, gmSV40 was shown to be a host range mutant the infectivity of which for human fibroblast cells is significantly higher than that of wild-type virus. This greater infectivity for human cells may allow us to extend the SV40 model system to studies of aberrant DNA replication in human cells with defects in DNA replication and repair.

RESULTS

Replication of gmSV40 in GM637 cells

Initial studies of gmSV40 DNA replication involved straight-forward comparisons with the replication of wild type SV40 in permissive CV-1 cells. GmSV40 is present in GM637 cells at levels that allow easy visualization by ethidium staining of the Hirt extracted viral DNA after agarose gel electrophoresis (Mitchell *et al.*, 1994). By dot-blot hybridization comparison with standard amounts of purified SV40 DNA, we estimate that GM637 cells contain 13,000 copies of SV40 per cell. This value is in good agreement with that obtained by Maulbecker (1989) of 10,000 copies per cell. By comparison, CV-1 cells infected with wild-type SV40 (strain 777) at an m.o.i. of 10:1 have ~192,000 copies per cell 36 h p.i. High-resolution agarose gel electrophoresis of pulse-labeled viral replication intermediates allows detection and resolution of many minor replication intermediates such as catenated daughter chromosomes and circular (head-to-tail) oligomers (Permana *et al.*, 1994; Snapka and Permana, 1996). Replication of episomal gmSV40 in GM637 cells and wild-type SV40 (strain 777) in CV-1 monkey kidney cells was compared by pulse-labeling of replicating DNA with [³H]Tdr, selective extraction of the viral DNA by the method of Hirt (1967), and separation of the deproteinized viral DNA by agarose gel electrophoresis. Pulse-labeled viral DNA separated by electrophoresis was visualized by gel fluorography (Fig. 1). Incorporation of label into gmSV40 DNA indicates that the episomal viral DNA normally found in GM637 cells is very actively replicating. A gmSV40 CV-1 stock was prepared by infecting CV-1 cells with medium from GM637 cells (gmSV40 media stock, see Methods for details of virus stocks). This stock was used to infect CV-1 cells for pulse-labeling and comparison with the replication of gmSV40 in GM637 cells (gmSV40 in CV-1, Fig. 1). The gmSV40 replicating in CV-1 cells is identical in size to that in GM637 cells, indicating that it still retains the A gene deletion reported to give gmSV40 a replication advantage in human cells (Maulbecker *et al.*, 1992). A faint band of more rapidly migrating form I DNA can be seen in front of the major form I band in this sample. This is the typical behavior of "variant" viral genomes the size of which is changed due to deletions and other DNA rearrangements during viral replication. If essential viral genes are inactivated by these rearrangements, as is often the case, they are defective variants the replication of which depends on wild-type viral genomes that can supply the missing functions in *trans*. Maulbecker noted the absence of variants in the episomal gmSV40 maintained in GM637 cells (Maulbecker, 1989), and our results confirm this observation. The presence of variants in the gmSV40-infected CV-1 cells suggests that their absence in GM637 cells is not a unique property of gmSV40, but we cannot rule out the possibility that prep-

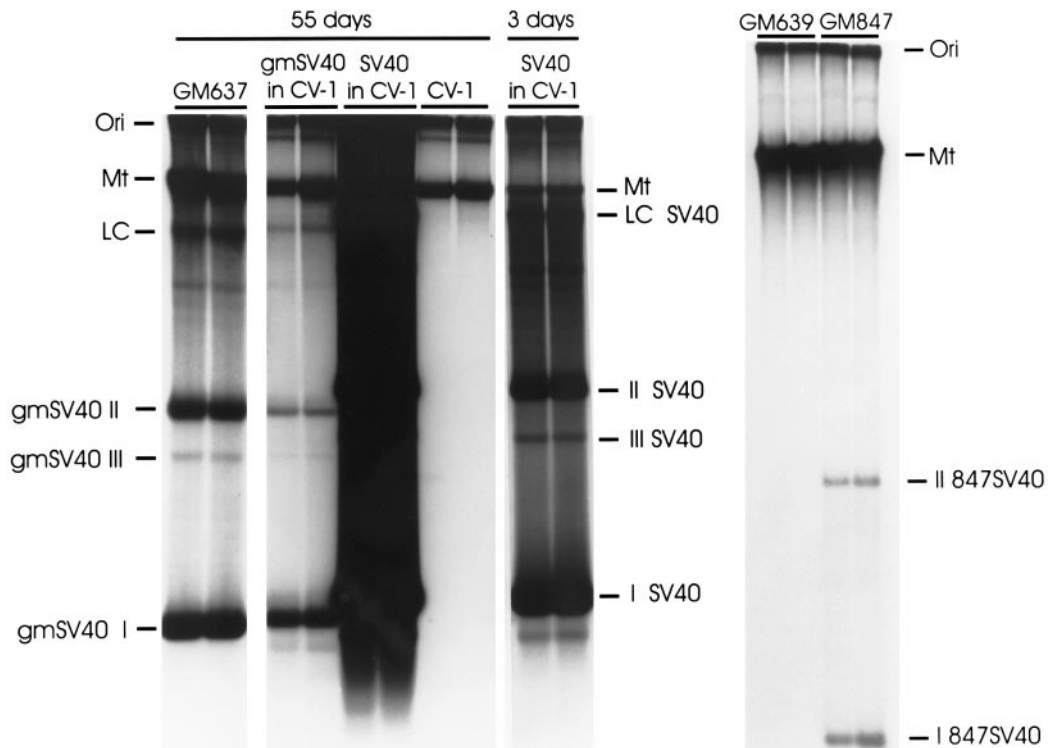


FIG. 1. Replication of endogenous gmSV40 in GM637 cells. Cells were labeled with [^3H]Tdr (250 $\mu\text{Ci/ml}$) for 30 min. Pulse-labeled viral DNA replication intermediates were purified and separated by agarose gel electrophoresis, and the gels were processed for tritium fluorography to visualize the pulse-labeled viral DNA replication intermediates on X-ray film. The fluorographic exposure of lanes 1–8 was 55 days and for lanes 9 and 10, 3 days. The long exposure was intended to detect variant viral genomes that arise due to recombinational events and that are often smaller than the major form. Variants are most easily detected as more rapidly migrating form I bands because that band is usually heavily labeled and because the region ahead of it is free of normal viral intermediates. All samples were run in duplicate. Lanes: GM637, gmSV40 normally present in GM637 cells; gmSV40 in CV-1, gmSV40 CV-1 stock-infected CV-1 cells (m.o.i. = 0.002 pfu/cell, based on a CV-1 cell plaque titer of 2×10^3 pfu/ml); SV40 in CV-1, wild-type SV40 (strain 777) from infected CV-1 cells (m.o.i. = 10 pfu/cell); CV-1, uninfected CV-1 cells; GM639, SV40-transformed cell line; GM847, another independently derived SV40-transformed human fibroblast line, previously reported not to contain episomal SV40 DNA. The gmSV40 CV-1 stock was prepared from CV-1 cells infected with medium conditioned by GM637 cells (see Methods for details of different viral stocks). Wild-type SV40 stock was $1 \times$ plaque purified. Variant viral genomes are seen as density (and sometimes as distinct bands) that migrate more rapidly than the dominant form I band. Ori, origin of electrophoresis; Mt, mitochondria DNA; LC, late Cairns replication intermediate (a “figure 8” late theta replication intermediate in which the terminal 5% of the genome remains unreplicated). Normal theta-form replication intermediates are distributed as a continuous smear between the form I bands and the LC bands. Form I (superhelical circular DNA), form II (nicked circular DNA), and form III (double-strand linear viral DNA) are indicated as I, II, and III, respectively. The identity of each form I, II, or III band is indicated as SV40 (wild-type SV40 strain 777), gmSV40 (resident episomal SV40 genomes in GM637 cells), and 847SV40 (resident SV40 genomes in GM847 cells). Because gmSV40 genomes have a significant deletion in the viral A gene, they migrate more rapidly than wild-type SV40 strains.

agation of the stocks in CV-1 cells has not selected for mutants with altered properties. Variants and viral defective genomes are favored by high multiplicity infection. When CV-1 cells are infected with wild-type SV40 at high m.o.i., a smear of unresolved variant genomes can be seen migrating more rapidly than the primary form I band (Fig. 1). Shorter exposure shows that a single variant dominates.

SV40-transformed GM639 cells also have been reported to contain episomal SV40 DNA (Hwang and Kucherlapati, 1980). However, another laboratory did not detect episomal SV40 in GM639 cells (Gish and Botchan, 1987), and we were not able to detect it (Fig. 1). In contrast, GM847 cells have been reported to be free of episomal SV40 (Hwang and Kucherlapati, 1983), yet we find significant levels of replicating SV40 (Fig. 1). The

episomal SV40 in GM847 cells appears to be the same size as that in GM637 cells as determined by agarose gel electrophoresis. We ordered the GM639 and GM847 cells specifically to test for episomal SV40 and took precautions to prevent cross-contamination with GM637 cells or wild-type virus. Both lines were kept isolated and were worked with separately in UV-preirradiated containment hoods. The work was done as rapidly as expansion of the lines after arrival in the laboratory allowed without intervening liquid nitrogen storage.

GM637 subclones are usually episome-free

GM637 cells were subcloned by plating at low dilution (10,000 cells per 10 cm plate) to ensure cellular homogeneity for planned studies and to determine whether

TABLE 1
Virus Stocks and Titers

Cell type:	CV-1		GM637d2
Assay type:	Plaque titer (p.f.u./ml)	Infective center (IU/ml)	Infective center (IU/ml)
Virus stock:			
Wild-type SV40 (777)	2.34×10^7	2.3×10^7	1.5×10^5
GmSV40 media stock	1	200	7×10^4
GmSV40 CV-1 stock	2×10^3	Not done	Not done
GmSV40 plaque stock	1.8×10^6	2.1×10^6	1.5×10^4

subclones might carry different levels of the episomal virus. In the case of mouse cell lines maintaining high-copy-number bovine papilloma virus genomes as stably replicating episomes, it has been possible to isolate high-copy-number subclones that are more prone to run-away episome replication (Ravnan and Cohen, 1995). The resulting colonies were isolated by glass cloning cylinders and removed by trypsinization. The clones then were expanded and studied for the presence of episomal SV40 DNA. Twenty one of the twenty-four clones isolated had no detectable gmSV40 DNA as determined by ethidium staining after Hirt extraction and agarose gel electrophoresis. The subcloning was done on the assumption, based on the literature, that the gmSV40 was maintained in GM637 cells by true episomal replication. Our hope was to isolate subclones containing very high levels of episomally replicating gmSV40. However, the gmSV40-positive subclones obtained had levels of episomal virus no higher than that of the original GM637 cell population. The high frequency of episome-free subclones suggested that the episomal virus in GM637 cells might represent a persistent, but inefficient lytic infection. One of the episome-free clones, GM637d2, was selected for further study.

Infective center assay

GM637 cells are known to produce low levels of active SV40 capable of lysing monolayers of permissive CV-1 cells (Maulbecker, 1989). GmSV40 and xpSV40 DNAs did not give plaques on CV-1 cells (Maulbecker *et al.*, 1992). Medium conditioned by GM637 cells formed minute plaques on CV-1 cells in ~27 days (compared with 12 days for wild-type SV40) and gave a titer of 1 pfu/ml (Table 1). The same medium gave 14 pfu/ml on Vero cells. GM637d2 cells did not form plaques when infected with medium from GM637 cells or with wild-type SV40 stocks. To determine whether plaque formation accurately represented the infectivity of gmSV40 on CV-1 and GM637d2 cells, we developed an infective center assay that does not depend on plaque formation (Methods). In brief, cell layers are transferred to membranes and lysed in place so that viral DNA binds and can be hybridized to probes. Infective centers were seen as small areas hy-

bridizing to the probe and were scored as infective units (IU). The infective center assay was validated with wild type SV40 on CV-1 cells. A wild-type SV40 stock that titered at 2.34×10^7 pfu/ml on CV-1 cells by the classical plaque assay gave a similar value of 2.3×10^7 IU/ml with the infective center assay (Table 1). This same stock gave a titer of 1.5×10^5 IU/ml when titered on GM637d2 cells with the infective center assay.

When medium from GM637 cells (gmSV40 media stock), with a titer of only 1 pfu/ml by plaque assay on CV-1 cells and 14 pfu/ml on Vero cells, was titered on CV-1 cells with the infective center assay, it was found to have 2×10^2 IU/ml, indicating that plaque formation on CV-1 cells does not give a good measure of the active viral titer (Table 1). When the same GM637 medium was titered on GM637d2 cells with the infective center assay, it was found to have a titer of 7×10^4 IU/ml (Fig. 2). For additional validation of assay, GM637 media stock was assayed on GM637d2 cells by limiting dilution. Serial dilutions of the medium were added to multiple small (35 mm) plates of confluent GM637d2 cells. After 7 days, the cells from each plate were passed to new plates (1:4), and these were allowed to reach confluence. At confluence the cells were Hirt extracted and the Hirt supernatants were deproteinized and processed for agarose gel electrophoresis as described under Methods. Viral DNA was detected by ethidium bromide staining. This method assumes that a single infective unit can reestablish a persistent gmSV40 infection in GM637d2 cells. The titer obtained by this limiting dilution method, 7×10^4 IU/ml, was identical to that obtained by the infective center assay.

Infection of GM637d2 cells with either gmSV40 or wild-type SV40

Episome-free GM637d2 cells were infected with different stocks of gmSV40 and wild-type SV40. In Fig. 3, GM637d2 cells were mock infected with fresh unused culture medium, infected with gmSV40 media stock (0.07 IU/cell), infected with gmSV40 plaque stock (0.015 IU/cell), or infected with wild-type SV40 777 stock (0.04 IU/cell). The m.o.i. given were based on the infective center titer obtained for the respective virus stocks on GM637d2 cells. At 7 days p.i., the cells were extracted by the Hirt procedure, and the Hirt supernatants were deproteinized and processed for agarose gel electrophoresis as described under Methods. No ethidium staining SV40 DNA bands were detected in the mock-infected GM637d2 subclone (Fig. 3, Ctrl, control). Viral DNA bands were detected in the GM637d2 cells infected with gmSV40 media stock (Fig. 3, GM637 media) and the GM637d2 cells infected with gmSV40 plaque stocks (Fig. 3, GMSV40 stock). The size of the gmSV40 DNA in both the cells infected with GM637-conditioned medium and stock prepared

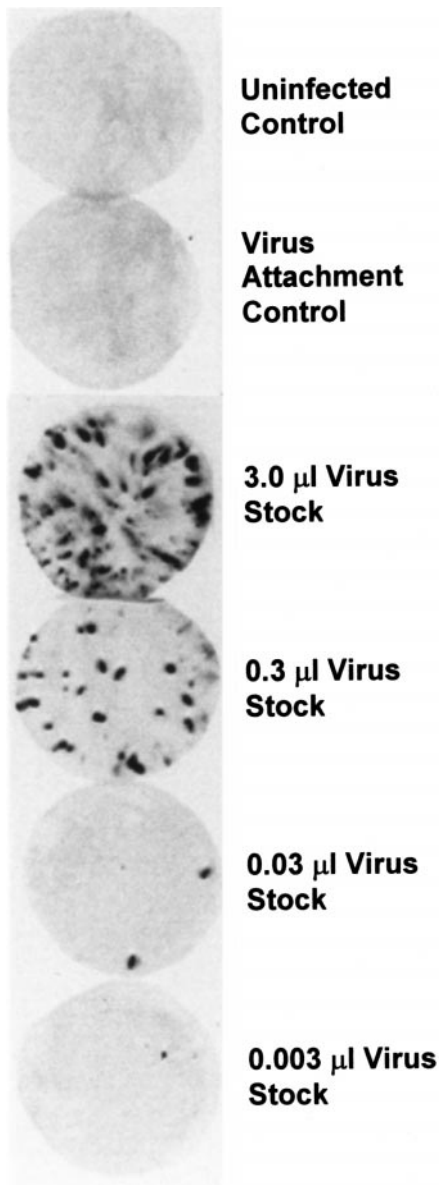


FIG. 2. Infective center assay. Confluent monolayers of GM637d2 cells were infected with 10-fold serial dilutions of gmSV40 media stock, then covered with agar containing medium with 10% FCS. At 7 days p.i., the agar was removed and the cell layer was picked up by allowing the cells to adhere to a dry nylon membrane disk (see Methods). The cells were lysed by floating the disk, cell side up, on Hirt lysing solution. The disk then was processed for filter hybridization with the SV40 nonspecific probe L443. Controls included uninfected cells and a virus attachment control that was processed immediately after virus attachment (3 μ l of virus stock) to show that signals were due to true infective centers and not to attached or internalized infecting virions. Ten-fold dilutions of the virus stock were used, with the volume applied to the cells being kept constant (300 μ l per 35-mm dish). The volumes indicated represent the volume of the original undiluted stock in each dilution.

in CV-1 cells is identical within the resolution of this gel, suggesting that the A gene deletion of gmSV40 has not been lost during passage in CV-1 cells.

Infection of GM637d2 cells with wild-type SV40 also results in significant levels of episomal viral DNA. The size of the wild-type virus is larger because it does not have the A gene deletion of gmSV40. This causes the DNA to migrate more slowly as shown by the form I and II bands. A Southern blot of the ethidium stained gel was hybridized with probes that are either specific for wild-type SV40 or nonspecific, detecting either wild-type or gmSV40 DNA. The location of the target sequences on the SV40 genome is indicated in Fig. 4. The SV40-specific probe, E321, spans the A gene deletion in gmSV40 genomes. The nonspecific probe, L443, covers the ag-nogene region shared by both wild-type SV40 and gmSV40. Both probes show that the mock-infected GM637d2 subclone is free of episomal viral DNA (Fig. 3). The nonspecific probe detects SV40 DNA in the GM637d2 cells infected by GM637-conditioned medium, gmSV40 stocks prepared in CV-1 cells, and wild-type SV40. The wild-type SV40-specific probe, targeted to the region deleted in gmSV40 genomes, detects wild-type SV40 only in the GM637d2 cells infected with wild-type SV40 viral stocks. This confirms the identity of the infecting virus in each case and shows that the gmSV40 retains the A gene deletion even when grown in CV-1 cells. The gmSV40 DNA and the wild-type SV40 DNA in infected GM637d2 cells is actively replicating, as shown by incorporation of [3 H]Tdr and agarose gel fluorography (Fig. 5).

When gmSV40 was used to reinfect GM637d2 cells, it was carried through multiple passages at high levels without apparent cytopathic effect. The reinfecting GM637d2 cells were indistinguishable from the original GM637 cells (Fig. 6A). The effect of m.o.i. on the level of virus in GM637d2 cells is shown in Fig. 6B. As increasing volumes of gmSV40 stock were added, increasing the m.o.i. from 3.5×10^{-3} to 3.5×10^{-2} IU/cell (stock titered on GM637d2 cells), the level of episomal virus in the cells increased progressively. In contrast, progressively higher m.o.i. infections of the GM637d2 cells with wild-type SV40, from 0.015 to 0.15 IU/cell (based on the infective center titer of the wild-type SV40 stock on GM637d2 cells), caused only a slight increase in the level of episomal viral DNA. These data show that the level of episomal virus replication is a function of m.o.i. for GM637d2 cells, suggesting that progressively more cells are infected. An approximately fourfold higher m.o.i. infection of GM637d2 cells with wild-type SV40, however, results in a lower level of virus replication that is not significantly increased by higher m.o.i. infection with wild-type virus. This may indicate that only a small fraction of GM637d2 cells can be infected with wild-type SV40 or that these human fibroblasts can only support wild-type SV40 DNA replication to a limited extent.

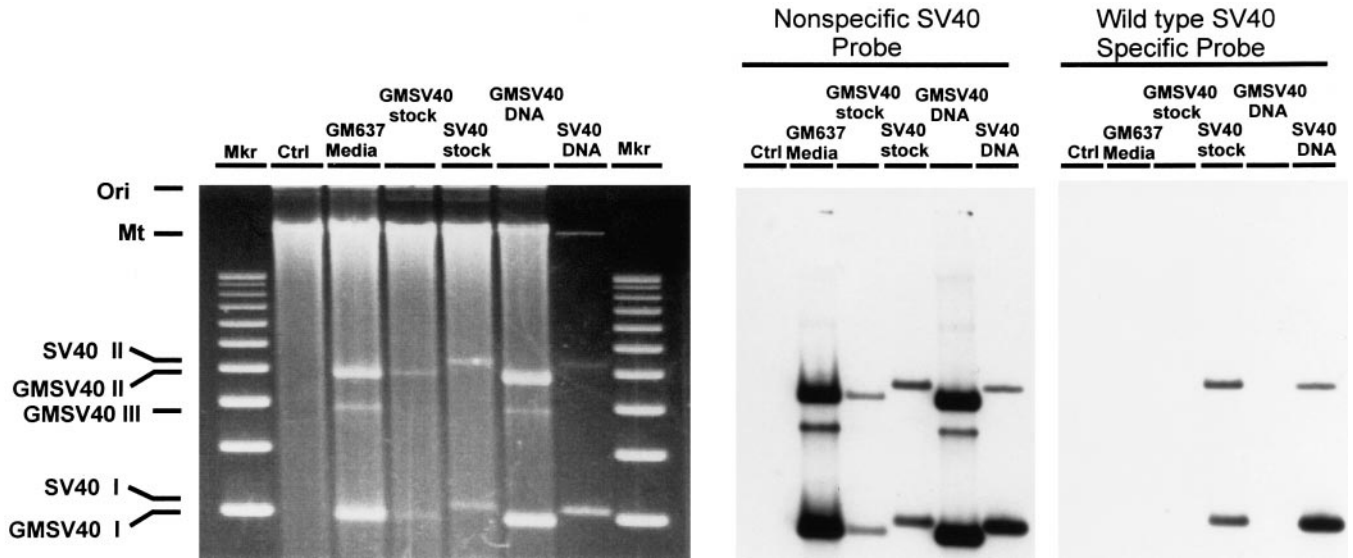


FIG. 3. Infection of GM637d2 cells with gmSV40 or wild-type SV40. The GM637d2 cell line was one of several episome-free subclones obtained from the original GM637 cell population. The episomal viral DNA was extracted, purified, and separated by agarose gel electrophoresis at 7 days p.i. The ethidium-stained gel is shown on the left, and Southern blots of this same gel are shown on the right. The blot was first probed with a nonspecific probe (L443) that hybridizes to the agnogene region common to both wild-type SV40 and gmSV40. The probe then was stripped off and the blot was reprobed with a wild-type SV40-specific probe (E321) against the sequences that are deleted in gmSV40. The location of the probes with respect to the SV40 genome are shown in Fig. 4. Mkr, Marker DNA (1-kb ladder, BRL); Ctrl, control (mock-infected GM637d2 cells); GM637 media, GM637d2 cells infected with gmSV40 media stock (0.07 IU/cell based on infective center titer of 7×10^4 pfu/ml on GM637d2 cells); GMSV stock, gmSV40 plaque stock, (0.015 IU/cell based on infective center titer 1.5×10^4 IU/ml on GM637d2 cells); SV40 stock, GM637d2 cells infected with wild-type SV40 stock (0.04 IU/cell based on infective center titer of 1.5×10^5 IU/ml on GM637d2 cells); GMSV DNA, episomal gmSV40 DNA purified from GM637 cells at passage 21; SV40 DNA, wild-type SV40 DNA isolated from infected CV-1 cells. The latter DNAs are routinely added as markers. Abbreviations are the same as in Fig. 1.

The integrated proviral SV40 copies do not contain the large T antigen deletion

Because the GM637d2 subclone was found to be free of episomal gmSV40 DNA, we used the specific probes

described above to determine whether the integrated proviral copies contained the A gene deletion found in gmSV40 genomes. Southern blots of restriction-digested GM637d2 genomic DNA with the wild-type SV40 specific probe E321 and the nonspecific SV40 probe L443 (Fig. 7) show that the integrated proviral SV40 copies in GM637d2 cells do not contain the large T antigen (A gene) deletion found in the episomal gmSV40 DNA. The absence of the A gene deletion in the integrated proviral copies means that the integrated copies do not give rise to episomal gmSV40 DNA in GM637 cells.

GM637 cells can be cured of episomal gmSV40 DNA by treatment with neutralizing SV40 antibody

If episomal gmSV40 in GM637 cells represents a persistent infection, it should be possible to cure the cells of episomal virus by neutralizing antibody. Goat anti-SV40 neutralizing antibody was added to medium at a ratio of 1:50. Goat serum was added to controls. The episomal gmSV40 was dramatically reduced by neutralizing antibody in the first passage (Fig. 8). There was no episomal virus detectable by Southern blot after the third passage while episomal virus remained constant in the controls treated with goat serum. When the cured cells were subsequently grown in the absence of neutralizing antibody, there was no return of the episomal virus (Fig. 8).

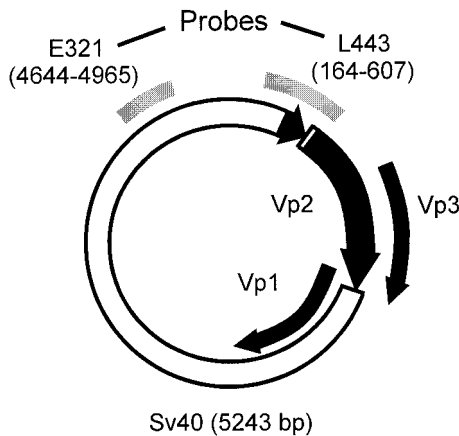


FIG. 4. Locations of SV40 specific and nonspecific probes on the SV40 genome. The map shows the SV40 genome with the late region genes VP1, VP2, VP3, and the agnogene indicated. The nonspecific probe L443 hybridizes to nucleotides 164-607 in the agnogene region common to both wild-type SV40 and gmSV40. The wild-type SV40 specific probe E321 is directed against nucleotides 4644-4965 and spans the region deleted in gmSV40.

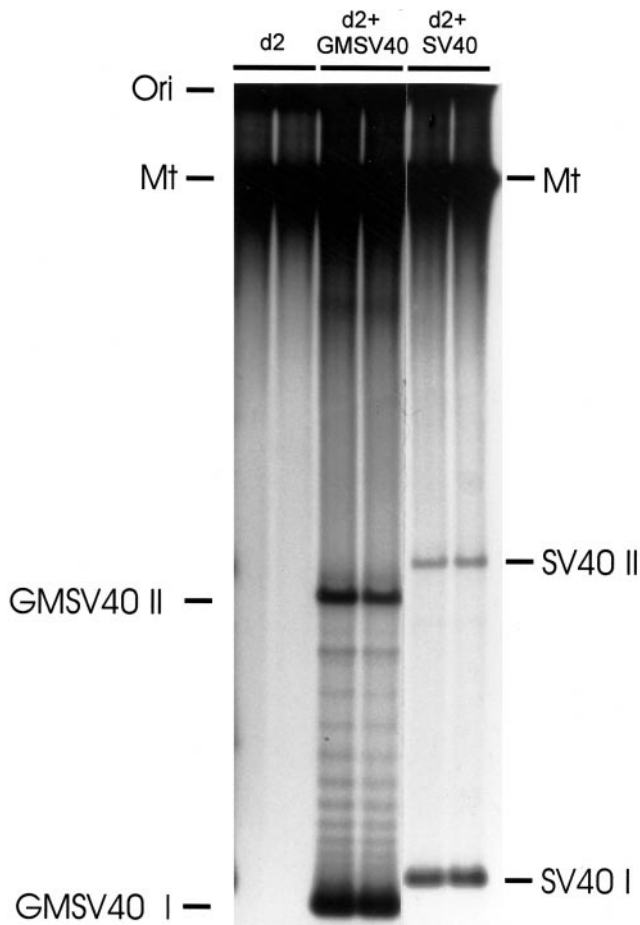


FIG. 5. Replication of gmSV40 and SV40 genomes in infected GM637d2 cells. GM637d2 cells were mock infected (d2), infected with wild-type SV40 stock (d2 + SV40) at an m.o.i. of 0.03 IU/cell, or infected with gmSV40 media stock (d2 + gmSV40) at an m.o.i. of 0.07 IU/cell. The m.o.i. values given are based on infective center titers on GM637d2 cells. Infected cells were pulse-labeled with [3 H]Tdr (250 μ Ci/ml) 36 h p.i. Pulse-labeled viral DNA was extracted, purified, and separated by agarose gel electrophoresis and visualized by gel fluorography. Abbreviations are the same as in Fig. 1.

DISCUSSION

GmSV40 represents a persistent lytic infection in GM637 cells

The rapid and complete curing of episomal gmSV40 in GM637 cells by neutralizing antibody shows that the presence of episomal virus in this line is due to a persistent lytic infection. The speed of curing, with a dramatic decrease in episomal virus in the first passage suggests that true episomal replication makes little or no contribution to maintenance of the episome. From these results, it is apparent that virus producing cells can survive for no more than three passages. Earlier work (Hwang, 1980) suggested that the chromosomally integrated SV40 copies on chromosome 8 could not give rise to episomal virus due to separation of the two copies by cellular DNA and a large deletion in one of the copies.

The demonstration that the chromosomal copies do not contain the A gene deletion of gmSV40 proves that the episomal virus cannot arise from the integrated copies by any rare recombinational event.

The frequent loss of episomal gmSV40 from subclones of GM637 cells suggests that reinfection is inefficient. This may be due to a combination of low numbers of infected cells, low burst size and inefficient attachment and entry into new cells. Evidence that early steps in attachment are limiting for SV40 infections of human cells has been reported (Ozer *et al.*, 1981). There is also evidence that infected cells in the GM637 population may be selectively lost at each passage. We have observed that levels of gmSV40 are relatively low in freshly confluent cells even after passage at a 1:4 split ratio. The levels of episomal virus continue to increase after the cells become confluent. Mitchell *et al.* reported a 100 times "amplification" of episomal gmSV40 in GM637 cells as they were held postconfluence (Mitchell *et al.*, 1994). On the basis of earlier literature indicating episomal replication of gmSV40 in GM637 cells, Mitchell *et al.* interpreted this as an escape of the plasmid from cellular replication control. A more likely explanation in light of the findings reported here is that infected cells are selectively lost at each passage and that the fraction of infected cells increases as a function of time after the passage as the surviving infected cells release active virus. Slow, inefficient reinfection may account for the homogeneous size of gmSV40 genomes maintained in GM637 cells. Amplification of defective virus genomes is thought to require coinfection with a defective and a wild-type virus. If the reinfection is very inefficient, this is an improbable event. However, defective virus can be amplified in cells the integrated viral copies of which encode functional large T antigen. Although the integrated viral copies in GM637 cells do not contain the large T antigen deletion characteristic of the episomal copies, it is not known if the large T antigen that they encode is functional for DNA replication.

Because the episomal gmSV40 in GM637 cells does not arise from the chromosomally integrated copies on chromosome 8, their consistent presence in GM637 cells is likely to represent a persistent infection originating from the transformation of the original GM037 human fibroblasts with wild-type SV40 stocks. GmSV40 may have been a variant present in the original virus stocks used for transformation. GmSV40 is not a true defective because it can replicate in CV-1 cells. The replication in CV-1 cells shows that gmSV40 is not dependent on a chromosomally integrated copy of SV40 for replication competent large T antigen. It is clear that the large T antigen produced by gmSV40 is replication competent. Episomal SV40 also has been found in the two cell lines GM639 (Hwang and Kucherlapati, 1980) and GM847 (this report). The episomal SV40 that we observe in GM847 cells is identical in size to gmSV40, suggesting the same

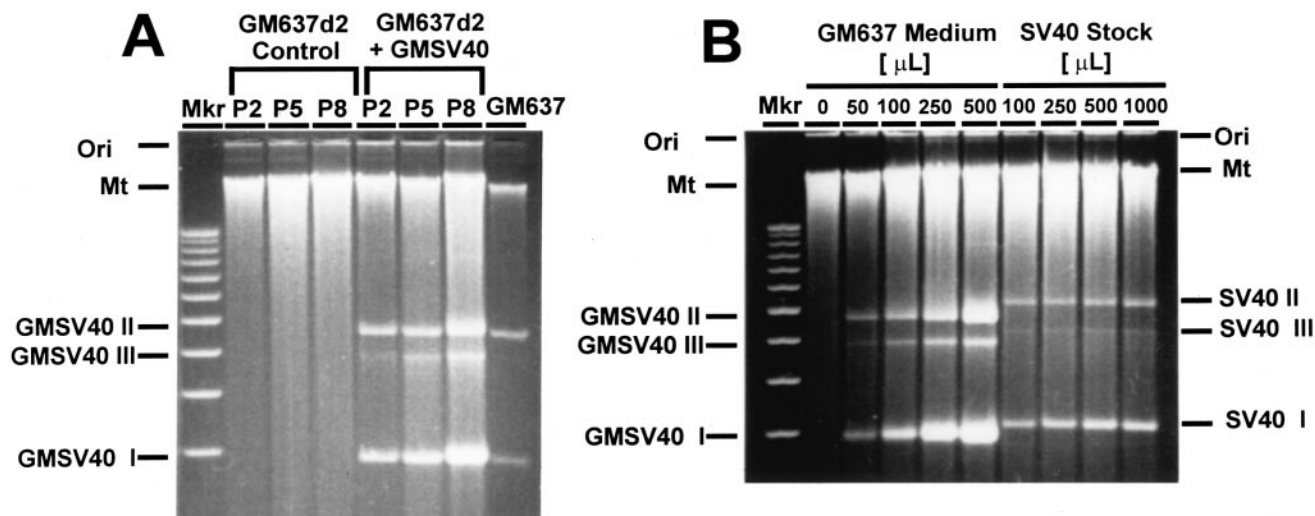


FIG. 6. Reestablishment of persistent infection and effects of m.o.i. (A) Reestablishment of gmSV40 in GM637d2 cells. GM637d2 cells were either mock infected (GM637d2 Control) or infected with GM637 medium stock at an m.o.i. of 0.07 IU/cell (GM637d2 + GMSV40), then passed for eight passages at a 1:4 split ratio. The free viral DNA was extracted by the Hirt method and separated by agarose gel electrophoresis. DNA was visualized by ethidium bromide staining. Episomal gmSV40 DNA extracted from early passage GM637 cells also was included (GM637). A 1-kb DNA ladder (BRL) was used as a marker. (B) Effect of m.o.i. on infection of GM637d2 cells by gmSV40 and wild-type SV40. Both GM637 media stock (7×10^4 IU/ml) and wild-type SV40 stock (1.5×10^5 IU/ml) were used to infect GM637d2 cells at different m.o.i.s. For GM637 media stock, 50 μ l corresponds to $\sim 3.5 \times 10^{-3}$ IU/cell and 500 μ l corresponds to 0.035 IU/cell. For the wild-type SV40 stock, 100 μ l corresponds to 0.015 IU/cell and 1000 μ l corresponds to 0.15 IU/cell. Viral titers for both gmSV40 and wild-type SV40 stocks were determined with the infective center assay on GM637d2 cells. Episomal viral DNA was extracted 7 days after infection, deproteinized, separated by agarose gel electrophoresis, and stained with ethidium bromide.

A gene deletion as seen in the gmSV40 and the xpSV40 of XP12ROSV cells. However, the detection of the episomal virus in each case is inconsistent, being observed by some investigators and not by others. We feel that there may be an explanation for this. If the episomal virus in these lines represents an inefficient persistent infection, it may be lost by plating at high cell dilution as the gmSV40 was lost from GM637 cells by plating at high dilution for subcloning. The probability of loss may be a function of the split ratio. There may be an increased probability of losing episomal virus at the thawing step after prolonged frozen storage of cells because there often is increased cell death at this step. GM637, GM639, and GM847 all originated in the laboratory of Dr. A.E. Greene (Hwang, 1980) and may have been transformed with the same viral stocks containing gmSV40 as a variant. The presence of an almost identical episomal SV40 in XP12ROSV cells may be due to contamination of that line or its precursor, XP12RO, during handling in a laboratory that also carried GM637 or a similar cell line.

Episomal polyomaviruses are also frequently observed in tumors and transformed cells (Lednický and Butel, 1999). In most cases, it is not known if the episomes represent products of rare excision events from integrated copies, true episomal replication, or persistent infections. It is possible that persistent SV40 infections may not be limited to cell-culture systems but also may play a role in tumorigenesis. Persistent SV40 infection has been shown to cause changes characteristic of transformation, such as increased cell density, increased

plating efficiency, and genetic instability as evidenced by high levels of chromosome aberration and aneuploidy (Norkin *et al.*, 1985). The pathogenesis of SV40 infections in humans warrants continued study.

Host range

SV40 is known not to cause cytopathic effects in some types of cells, including Rhesus monkey and human kidney (Hsiung and Gaylord, 1961). Because SV40 does not cause cytopathic effects in GM637d2 cells, an infective center assay was required. For wild-type SV40 titrated on CV-1 cells, plaque forming units are equal to infective units as determined by our infective center assay. The titer of gmSV40 on episome-free GM637d2 cells as determined by limiting dilution is also the same as the titer determined by the infective center assay. These experiments validate the infective center assay, which does not depend on cytopathic effect (visible plaque formation). Medium conditioned by GM637 cells gave a titer of only 1 pfu/ml in the classical plaque assay on CV-1 cells, but gave a titer of 200 IU/ml on CV-1 cells with the infective center assay. Visible plaques account for only 0.5% of infective centers. This suggests that the visible plaques are due to rare cytopathic variants that arise during the infection of the CV-1 cells. When medium conditioned by GM637 cells is added to CV-1 cells in culture, complete lysis of the cell layer occurs in 27 days. This complete lysis is probably due to the dominant effect of these rare cytopathic variants when they are not

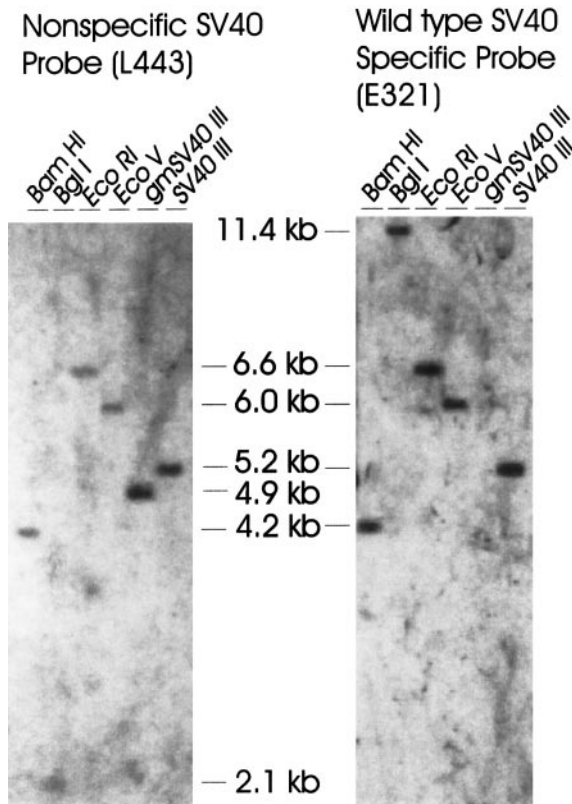


FIG. 7. Absence of the gmSV40 A gene deletion in the chromosomally integrated SV40 copies of GM637 cells. GM637d2 genomic DNA was digested with *Bam*HI, *Bgl*I, *Eco*RI, or *Eco*RV. The restriction-digested genomic DNA was separated on an agarose gel, transferred to a nylon membrane filter, and hybridized to the wild-type SV40 specific and nonspecific probes E321 and L443 (Fig. 4). Linearized wild-type SV40 DNA (SV40 III) and gmSV40 DNA (gmSV40 III) were included as controls.

restricted to their sites of origin by the agar overlay involved in plaque or infective center assays.

The infective center assay for gmSV40 media stock on GM637d2 cells gave a titer of 7×10^4 IU/ml, more than two orders of magnitude higher than the infective center titer of this same media stock on CV-1 cells (200 IU/ml). This increased infectivity for the human fibroblasts indicates that gmSV40 is a host range mutant. In contrast, wild-type SV40 strain 777 stocks with a titer of 2.3×10^7 (pfu/ml and IU/ml on CV-1 cells) gave a titer of 1.5×10^5 IU/ml on GM637d2 cells, indicating higher infectivity for CV-1 cells than for human cells. When gmSV40 stocks were prepared on CV-1 cells, followed by plaque purification on CV-1 cells, and then a final preparation of gmSV40 viral stock on CV-1 cells with the plaque isolate (three cycles of growth in CV-1), the resulting stock had a titer of 1.8×10^6 pfu/ml by a classical plaque assay on CV-1 cells, an almost identical titer of 2×10^6 IU/ml by infective center assay on CV-1 cells and 1.5×10^4 IU/ml on GM637d2 cells. It is apparent that selection of a cytopathic variant of gmSV40 by plaque isolation on CV-1 cells and repeated passage through CV-1 cells resulted

in a virus stock in which, as with wild-type SV40 stocks, plaque forming units equal infective centers. For gmSV40 media stocks (medium conditioned by GM637 cells), the ratio of infective center titer on CV-1 cells to infective center titer on GM637d2 cells is ~ 0.003 , whereas this same ratio is 133 for the gmSV40 plaque isolate stocks prepared on CV-1 cells. This is roughly a 44,000-fold increase in the ratio of infectivity resulting from three passages through CV-1 cells.

The size of gmSV40 DNA did not change as a result of repeated passage through CV-1 cells, indicating that the A gene deletion responsible for its small size was not lost during the changes in cytopathicity and infectivity for CV-1 cells. The changes involved in host range and cytopathic effect may be due to small changes such as point mutations in other regions of the viral genome. Host range mutations map in the SV40 large T antigen C-terminal domain and are known to be involved in virion assembly, but additional virion assembly mutations map in the N-terminal domain of large T antigen (Spence and Pipas, 1994). These N-terminal virion assembly mutations map in the J domain, which is shared by SV40 large T antigen and small t antigen. The A gene deletion of gmSV40 also falls in the J-domain region and prevents small t antigen synthesis by deletion of the large T antigen/small t antigen splice site (Maulbecker *et al.*, 1992). Alterations in the J domain caused by the gmSV40 A gene deletion may interact in a complex way with mutations in other regions of the viral genome to affect virion assembly in different host backgrounds. Virion assembly also is affected by mutations in virion genes (Behm *et al.*, 1988; Ng and Bina, 1984), and a host range determinate in the viral late region has specifically been reported to affect growth of SV40 in human cells (O'Neill *et al.*, 1990). Because the gmSV40 A gene deletion has been shown to increase viral DNA replication in human cells while decreasing replication in monkey cells (Maulbecker *et al.*, 1992), host range effects involving DNA replication and transcription also may be involved.

Deletions in the early gene region also have been reported in human tumor-derived SV40 genomes (Carbone *et al.*, 1996; Tevethia *et al.*, 1998). It is interesting that one of them exhibits a deletion similar to the deletion in the gmSV40 genome (Carbone *et al.*, 1996). This SV40 sequence was amplified from human mesothelioma tumor tissue and harbors a deletion in the first intron of the early mRNA (4646–4897). Wild-type large T antigen and truncated small t antigen are expected to be expressed. Because the deletion in gmSV40 is also at this region (4643–4966), this region may play a role in host range modulation as well as persistent infection. Maulbecker *et al.* provided evidence that the large T antigen deletion in gmSV40 increases viral DNA replication in human cells (Maulbecker *et al.*, 1992). However, Tevethia *et al.* presented evidence that a large T antigen deletion in the choroid plexus tumor-derived SV40 genome B6SVCP

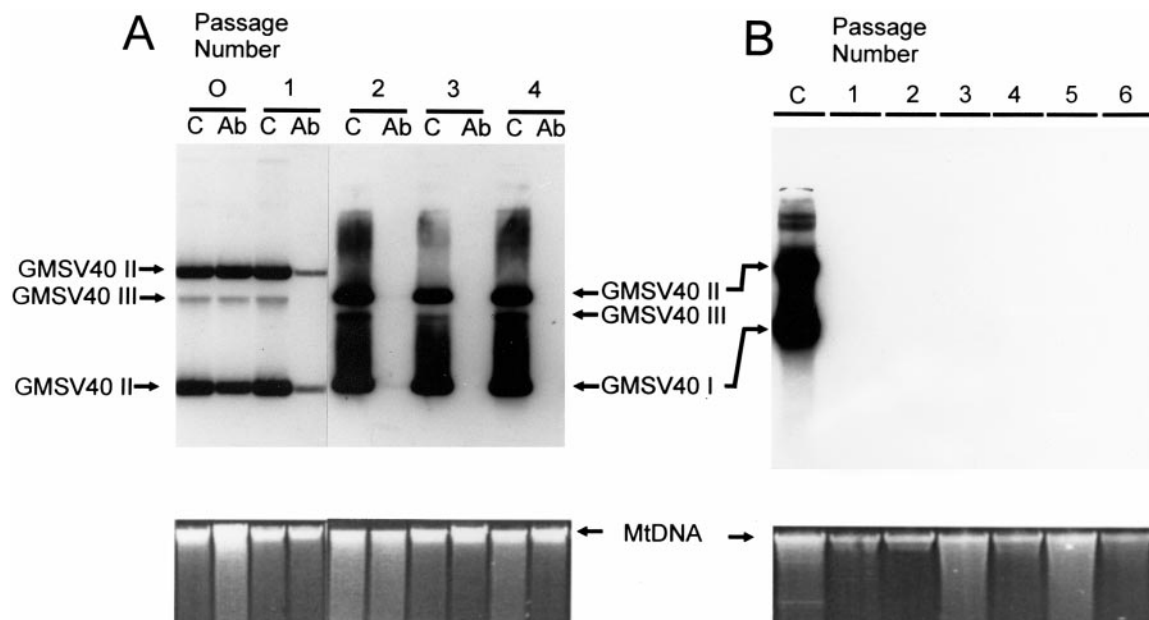


FIG. 8. Curing GM637 cells of episomal gmSV40 DNA with neutralizing SV40 antibody. (A) GM637 cells were passed in 60-mm culture plates at 1:4 split ratios either in medium supplemented with goat serum (control, C) or medium supplemented with neutralizing SV40 antibody (Ab). The passage number is shown above. GmSV40 form I, II, and III DNA are indicated as detected by Southern blotting with the SV40 nonspecific probe L443. Ethidium staining of the gel (mitochondrial DNA) is indicated below to show even loading. Passages 2–4 are more heavily exposed to bring up faint SV40 DNA bands in the antibody treated samples (when present). (B) Continued passage of GM637 cells cured of episomal gmSV40 DNA by treatment with neutralizing antibody. Antibody was not included in these passes. The control, C, is the original, untreated GM637 cell line. The experiment was very heavily exposed to detect the faintest traces of episomal viral DNA.

cl.4 affects large T antigen antigenicity and possibly host immune surveillance (Tevethia *et al.*, 1998). The screening of more tumor associated SV40 sequences with PCR primer sets designed to amplify the large T antigen intron region might provide some insight of these hypotheses.

The results in Fig. 6 show that increasing the multiplicity of infection of GM637d2 cells with gmSV40 results in progressively higher levels of episomal virus replication. This suggests that more cells are infected at higher m.o.i. In contrast, increasing the multiplicity of infection with wild-type virus resulted in only a relatively low and roughly constant level of viral replication. This may be due either to saturation of a limited subpopulation of the human cells that are capable of being infected or to some limit on the level of virus replication that these human fibroblasts can support.

The results reported here show that plaque assay on CV-1 cells does not necessarily reflect infectivity on CV-1 cells, especially in the case of host range mutants. Because infectivity and cytopathic effect can vary independently, plaque assays in general may be unsuitable for studies of host range. The use of an infective center assay that is independent of cytopathic effect allows quantitative studies of host range on any permissive or semipermissive cell type.

The finding that GM637 cells sustain a persistent SV40 infection with high levels of viral replication raises cautionary note for their use in studies of DNA replication

and repair. However, GM637 cells free of the episomal gmSV40 can be obtained by subcloning or by antibody treatment. The presence of structurally similar episomal viruses in other SV40-transformed cell lines such as XP12ROSV suggests that these lines may also have persistent virus infections. The increased infectivity of gmSV40 for human cells may facilitate the use of SV40 as a probe for aberrant DNA replication in human genetic instability syndromes and other human cell lines with defects in DNA replication.

METHODS

Cell culture, subcloning, and virus infection

Cell lines. GM637, GM847, and GM639 cells were obtained from the NIGMS Human Genetic Mutant Cell Repository. They were grown in a 5% CO₂ atmosphere at 37°C in modified Eagle's medium (MEM, Gibco) or Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), and penicillin/streptomycin. CV-1 monkey kidney cells were obtained from the American Type Culture Collection and were maintained in MEM supplemented with 10% calf serum and penicillin/streptomycin. Vero cells were a gift of Dr. Deborah Parris, Ohio State University.

GM637 cells were subcloned by seeding cells at low density (10,000 cells per 100 mm plate), and grown in DMEM supplemented with 20% FCS. Well-separated col-

onies of ~20 cells were isolated by glass cloning rings and removed by trypsinization and transfer to wells in a 96-well plate. The FCS was reduced to 10% when the clones were expanded. The episome status of each clone was determined by Southern blot hybridization with SV40 probes.

Virus stocks. Stocks of gmSV40 were prepared in three different ways. Some experiments were done with gmSV40 stock, consisting of medium conditioned by GM637 (gmSV40 media stock). To ensure consistency, a single batch of GM637-conditioned medium was frozen in aliquots and used for all the experiments with gmSV40 media stock. A second gmSV40 stock was made by infecting confluent CV-1 cells with medium from GM637 cells. The stock was harvested when the cells showed 90% cytopathic effect (gmSV40 CV-1 stock). A well-isolated plaque from the plaque assay of the gmSV40 CV-1 stock was used to prepare a plaque lysate, which was in turn used to infect CV-1 cells to prepare a 1× plaque-purified CV-1 stock (gmSV40 plaque stock). The wild-type SV40 strain 777 stock (SV40 stock) was prepared from a plaque lysate as described (Turler and Beard, 1985). SV40 777 forms large clear plaques on CV-1 cells in 12–15 days, whereas gmSV40 makes minute plaques in ~27 days. These stocks were titered by plaque assay on CV-1 cells and/or by infective center assay on CV-1 cells and on episome-free GM637d2 cells. The virus titers obtained with these assays are shown in Table 1.

Isolation and analysis of viral DNA

Experiments were done on cells grown in 35-mm cell-culture plates. Replicating DNA was pulse-labeled with [³H]Tdr (250 μCi/ml). Experiments were stopped by replacing the labeling medium with Hirt lysing fluid (Hirt, 1967). Viral DNA was selectively extracted by the Hirt method (Hirt, 1967) and prepared for electrophoresis by proteinase K digestion and chloroform-isopropanol extraction. After ethanol precipitation, the DNA was briefly dried under vacuum, then analyzed by one-dimensional submarine gel electrophoresis and gel fluorography (Snapka *et al.*, 1991).

Infective center assay

Ten-fold serial dilutions of viral stocks were made using serum-free medium, and 0.3-ml aliquots of these diluted stocks were used to infect confluent cell monolayers in 35-mm culture plates (1 h, 37°C, with gentle shaking every 15 min). One milliliter of medium with 10% FCS then was added and the cells were left at 37°C for 15 h. The medium then was drawn and replaced with 0.9% agar containing DMEM with 10% FCS. Five to 7 days p.i., the agar was gently removed and the cell monolayer was gently rinsed twice with warm PBS. The PBS was drawn off and a dry 35-mm nylon filter disk (Zetabind, AMF No. P899-02-045SP) was applied directly to the cell

layer. The filter was lifted, bringing the cell layer with it, and was floated, cell side up, on 150 μl of Hirt lysis fluid in a small culture dish (5 min). The membrane, with cell side up, was gently transferred to dry paper towels that removed most of the Hirt lysing solution by capillary action. Filters were air dried, then transferred to 150 μl of denaturing solution (0.5 N NaOH, 1.5 M NaCl, 3 min). After subsequent drying on paper towels, the filters were transferred to 150 μl of neutralizing solution (1 M Tris-HCl, pH 7.4, 1.5 M NaCl) in a small plate for 5 min. After air drying on paper towels, the filters were baked at 80°C for 2 h in a vacuum oven, then probed with the nonspecific SV40 probe L443 as described below.

Southern blotting, preparation of probes, and filter hybridization

Probes for hybridization were synthesized by PCR in the presence of digoxigenin-labeled dUTP. Probe L443 (nonspecific SV40 probe) was made using primer set 1 (5'-GGGACTTTCACACCTG-3' and 5'-ACACAGTAGCAATTAGGTCC-3') and Probe E321 (wild-type SV40 specific) was made with primer set 2 (5'-GCTTTAAATCTCTGTAGGTAG-3' and 5'-AAATATGCTCATCAACCTG-3'). The probes were amplified using the Boehringer Mannheim PCR Dig Probe Synthesis kit (No. 1636090). Each 50 μl PCR labeling reaction contained 1–2 ng of cloned SV40 DNA (pUC19-SV) as template, the digoxigenin-labeled dUTP and dNTP mixture provided in the kit. After an initial denaturing step (95°C, 3 min), 25–30 cycles of amplification were carried out (95°C, 30 s; 60°C, 45 s; 72°C, 60 s). The PCR product was separated on a 1.2% agarose gel, visualized by ethidium staining and purified using a QIAGEN QIAquick gel Extraction kit (No. 28704) as described by the manufacturer. The probe was diluted to a concentration of 1–5 ng/μl and stored at –20°C until use. The hybridization procedures were conducted according to Boehringer Mannheim's DIG system user Guide for filter hybridization (available from Boehringer Mannheim on request). The membrane was pre-wetted with 2× SSC for 5 min before prehybridization.

For Southern blots, either restriction-enzyme-digested genomic DNA or viral DNA was separated by agarose gel electrophoresis and transferred to nylon membranes as described (Sambrook *et al.*, 1989). The filters then were placed in either 11 × 23 cm plastic bags with 50 ml of prehybridization solution or in 11 × 11 cm plastic bags with 25 ml of prehybridization solution. For the infective center assay (above), eight 35-mm nylon filter disks were processed at a time. The disks were placed in 11 × 11 cm plastic bags with 25 ml of prehybridization solution [5× SSC, 0.1% sarkosyl, 0.02% SDS, and 1% Boehringer blocking solution (Boehringer Mannheim, No. 1096176)]. The prehybridization was done at 65°C for 2 h. The DIG-labeled probe was denatured by boiling for 10 min, then chilled on ice for 10 min. Hybridization solution was

prepared by adding denatured probe to freshly prepared prehybridization solution to a final concentration of 10–20 ng/ml. At least 10 ml of hybridization solution was required for each 11 × 11 cm bag and 20 ml for each 11 × 23 cm bag. Hybridization was carried out at 65°C for 16–18 h. The membrane then was washed for 15 min at room temperature in 2× wash buffer (2× SSC, 0.1% SDS), followed by a 15 min wash at room temperature in 0.5× wash buffer (0.5× SSC, 0.1% SDS), and then two 15-min washes in 0.5× wash buffer at 65°C. Detection was done at room temperature using the CDP-Star detection kit as described by the supplier (Boehringer Mannheim, No. 1685627). Briefly, the membrane was equilibrated in 1× maleic acid buffer (0.1 M maleic acid, pH 7.5, 0.15 M NaCl) for 5 min, followed by 1% blocking solution (Boehringer Mannheim, No. 1096176) for 30 min. The anti-DIG antibody conjugated to alkaline phosphatase (Boehringer Mannheim, No. 1093274) was added to the blocking solution at a dilution of 1:10,000–1:20,000. The membrane then was incubated with the antibody-containing blocking solution for 30 min. Unbound antibody was removed by washing the membrane twice (15 min each time) with 1× maleic acid buffer. The membrane was then equilibrated for 5 min with 1× detection buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl) and diluted (1:1000) CDP-Star substrate was added. The membranes were incubated with the substrate in sealed plastic bags for 5 min, then exposed to X-ray film for times ranging from 5 s to 10 min to obtain optimal exposures.

SV40 neutralizing antiserum treatment

GM637 was plated in 60-mm plates at a cell density of 3×10^6 cells per plate in medium supplemented with either goat SV40 neutralizing antiserum (titer: 1:100, Whitaker Bioproducts, No. 30-190H) or goat serum (BRL). Goat SV40 neutralizing antiserum was included in the culture medium in 1:50 ratio (60 μ l antiserum in 3 ml culture medium), and the culture medium was changed every day to maintain enough active antibody to neutralize newly released virus. For control plates, goat serum was added in the culture medium, and the medium also was changed every day to mimic neutralizing antibody treatments. The cells were passed every 3–4 days in a split ratio of 1 to 4. After six passages with neutralizing antiserum, the cells were maintained without neutralizing antiserum for another six passages. During these six passages, the cells were passed as a normal culture without changing medium every day. The viral DNA was isolated from cells at each passage by the Hirt extraction procedure, separated by agarose gel electrophoresis and transferred to nylon membrane for Southern blotting with the SV40 nonspecific probe L443.

ACKNOWLEDGMENTS

We thank Dr. Deborah Parris and Dr. Marshall Williams for reviewing the manuscript. This work was supported by Public Health Service

Grant CA-60914 to R.M.S. and, partially, Grant P30 CA-16058 to The Ohio State University Comprehensive Cancer Center.

REFERENCES

- Behm, M., Lowman, H., Ng, S.-C., and Bina, M. (1988). Analysis of temperature-sensitive mutations in the simian virus 40 gene encoding virion protein 1. *Proc. Natl. Acad. Sci. USA* **85**, 9421–9425.
- Carbone, M., Rizzo, P., Procopio, A., Giuliano, M., Pass, H. I., Gebhardt, M. C., Mangham, C., Hansen, M., Malkin, D. F., Bushart, G., Pompetti, F., Picci, P., Levine, A. S., Bergsagel, J. D., and Garcea, R. L. (1996). SV40-like sequences in human bone tumors. *Oncogene* **13**, 527–535.
- Denissenko, M. F., Venkatachalam, S., Yamasaki, E. F., and Wani, A. A. (1994). Assessment of DNA damage and repair in specific genomic regions by quantitative immuno-coupled PCR. *Nucleic Acids Res.* **22**, 2351–2359.
- Domoradzki, J., Pegg, A. E., Dolan, M. E., Maher, V. M., and McCormick, J. J. (1984). Correlation between O⁶-methylguanine-DNA-methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Carcinogenesis* **5**, 1641–1647.
- Dusinska, M., and Slamenova, D. (1992). Application of alkaline unwinding assay for detection of mutagen-induced DNA strand breaks. *Cell Biol. Toxicol.* **8**, 207–216.
- Fritz, G., and Kaina, B. (1992). Genomic differences between O⁶-methylguanine-DNA methyltransferase proficient (Mex+) and deficient (Mex-) cell lines: Possible role of genetic and epigenetic changes in conversion of Mex+ into Mex-. *Biochem. Biophys. Res. Commun.* **183**, 1184–1190.
- Gish, W. R., and Botchan, M. R. (1987). Simian virus 40-transformed human cells that express large T antigens defective for viral DNA replication. *J. Virol.* **61**, 2864–2876.
- Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**, 365–369.
- Hsiung, G.-D., and Gaylord, W. D. (1961). The vacuolating virus of monkeys. I. Isolation, growth characteristics, and inclusion body formation. *J. Exp. Med.* **114**, 365–369.
- Hwang, S.-P. (1980). "Localization, and organization of integrated simian virus 40 genomes in transformed human cells." Ph.D. Thesis, Princeton University, Princeton, NJ.
- Hwang, S.-P., and Kucherlapati, R. (1980). Localization and organization of integrated simian virus 40 sequences in a human cell line. *Virology* **105**, 196–204.
- Hwang, S.-P., and Kucherlapati, R. (1983). Events preceding stable integration of SV40 genomes in a human cell line. *Somat. Cell Genet.* **9**, 457–468.
- Lednický, J. A., and Butel, J. S. (1999). Polyoma viruses and human tumors: A brief review of current concepts and interpretations. *Front. Biosci.* **4**, 153–164.
- Maulbecker, C. (1989). "In vivo and in vitro replication studies of mutant SV40 virus from transformed human cells." Ph.D. Thesis, University of California, Berkeley.
- Maulbecker, C., Mohr, I., Gluzman, Y., Bartholomew, J., and Botchan, M. (1992). A deletion in the simian virus 40 large T antigen impairs lytic replication in monkey cells in vivo but enhances DNA replication in vitro: New complementation function of T antigen. *J. Virol.* **66**, 2195–2207.
- Mitchell, D. L., Cleaver, J. E., and Lutze, L. H. (1994). Excision of cyclobutane dimers in genomic and episomal DNA in human cells. *Photochem. Photobiol.* **60**, 221–224.
- Neufeld, D. S., Ripley, S., Henderson, A., and Ozer, H. L. (1987). immortalization of human fibroblasts transformed by origin-defective simian virus 40. *Mol. Cell. Biol.* **7**, 2749–2802.
- Ng, S.-C., and Bina, M. (1984). Temperature-sensitive BC mutants of simian virus 40: block in virion assembly and accumulation of capsid-chromatin complexes. *J. Virol.* **50**, 471–477.
- Norkin, L. C., Steinberg, V. I., and Kosz-Vnenchak, M. (1985). Human

- glioblastoma cells persistently infected with simian virus 40 carry nondefective episomal viral DNA and acquire the transformed phenotype and numerous chromosomal abnormalities. *J. Virol.* **53**, 658–666.
- O'Neill, F. J., Carney, H., and Hu, Y. (1998). Host range analysis of simian virus 40, BK virus and chimaeric SV40/BKV: Relative expression of large T antigen and Vp1 in infected and transformed cells. *Dev. Biol. Stand.* **94**, 191–205.
- O'Neill, F. J., and Carroll, D. (1981). Amplification of papovavirus defectives during serial low multiplicity infections. *Virology* **112**, 800–803.
- O'Neill, F. J., Xu, X. L., and Miller, T. H. (1990). Host range determinant in the late region of SV40 and RF virus affecting growth in human cells. *Intervirology* **31**, 175–187.
- Ozer, H. L., Slater, M. L., Dermody, J. J., and Mandel, M. (1981). Replication of simian virus 40 DNA in normal human fibroblasts and in fibroblasts from xeroderma pigmentosum. *J. Virol.* **39**, 481–489.
- Permana, P. A., Ferrer, C. A., and Snapka, R. M. (1994). Inverse relationship between catenation and superhelicity in newly replicated simian virus 40 daughter chromosomes. *Biochem. Biophys. Res. Commun.* **201**, 1510–1517.
- Ravnan, J.-B., and Cohen, S. N. (1995). Transformed mouse cell lines that consist predominately of cells maintaining bovine papilloma virus at high copy number. *Virology* **213**, 526–534.
- Royer-Pokora, B., Peterson, W. D., and Haseltine, W. A. (1984). Biological and biochemical characterization of an SV40-transformed xeroderma pigmentosum cell line. *Exp. Cell. Res.* **151**, 408–420.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning," 2 Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Schwartz, J. L., Banda, M. J., and Wolff, S. (1982). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces sister-chromatid exchanges and delays in cell progression in Chinese hamster ovary and human cell lines. *Mutat. Res.* **92**, 393–409.
- Simon, L., Hazard, R. M., Maher, V. M., and McCormick, J. J. (1981). Enhanced cell killing and mutagenesis by ethylnitrosourea in xeroderma pigmentosum cells. *Carcinogenesis* **2**, 567–570.
- Snapka, R. M., Ferrer, C. A., Sun, N. J., and Cassady, J. M. (1996). "The SV40 Replicon Model for Analysis of Anticancer Drugs" (R. M. Snapka, Ed.), pp. 127–148. Academic Press, San Diego.
- Snapka, R. M., and Permana, P. A. (1993). SV40 DNA replication intermediates: analysis of drugs which target mammalian DNA replication. *BioEssays* **15**, 121–127.
- Snapka, R. M., and Permana, P. A. (1996). "The SV40 Replicon Model for Analysis of Anticancer Drugs" (R. M. Snapka, Ed.), pp. 167–186. Academic Press, San Diego.
- Snapka, R. M., Permana, P. A., Marquit, G., and Shin, C.-G. (1991). Two-dimensional agarose gel analysis of simian virus 40 DNA replication intermediates. *Methods Companion Methods Enzymol.* **3**, 73–82.
- Spence, S. L., and Pipas, J. M. (1994). SV40 large T antigen functions at two distinct steps in virion assembly. *Virology* **204**, 200–209.
- Sweet, B. H., and Hilleman, M. R. (1960). The vacuolating virus, SV40. *Proc. Soc. Exp. Biol. Med.* **105**, 420–427.
- Tevethia, S. S., Mylin, L., Newmaster, R., Epler, M., Lednicky, J. A., Butel, J. S., and Tevethia, M. J. (1998). Cytotoxic T lymphocyte recognition sequences as markers for distinguishing among tumour antigens encoded by SV40, BKV and JCV. *Dev. Biol. Stand.* **94**, 329–339.
- Tooze, J. (1981). "DNA Tumor Viruses." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Turler, H., and Beard, P. (1985). "Virology, A Practical Approach" (B. W. J. Mahy, Ed.), pp. 169–192. IRL Press, Oxford.
- Woo, S. H., Sun, N. J., Cassady, J. M., and Snapka, R. M. (1999). Topoisomerase II inhibition by aporphine alkaloids. *Biochem. Pharmacol.* **57**, 1141–1145.
- Wright, J. A., Keegan, K. S., Herendeen, D. R., Bentley, N. J., Carr, A. M., Hoekstra, M. F., and Concannon, P. (1998). Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. *Proc. Natl. Acad. Sci. USA* **95**, 7445–7450.
- Zouzas, D., Jha, K. K., Mulder, C., Basilico, C., and Ozer, H. L. (1980). Human fibroblasts transformed by the early region of SV40 DNA. Analysis of "free" viral DNA sequences. *Virology* **104**, 439–453.